Supplementary Information

Extensive Methods

Animals

All animal experiments were conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, as well as the German law for the welfare of animals. Mice were housed in a SPF facility with free access to chow and water and a 12h day/night cycle. Breeding and genotyping was done according to standard procedures. *Rictor fl/fl* mice have been described previously(1) and were crossed to *KspCre*(2) animals and to the reporter strain mT/mG where indicated (3). Mouse genetic background was C57Bl6/N and controls consisted of either *Rictor fl/fl* (whole animal experiments) or *KspCre*mT/mG* (patch-clamp experiments) animals. Littermate controls were used wherever possible.

Renal excretion

To study renal excretion, all mice were placed into metabolic cages (Tecniplast, Hohenpeisenberg, Germany) for two days on control diet (C1000, measured Na^+ content 117 µmol/g, K^+ content 182 µmol/g, Altromin, Lage, Germany) and tap water (measured Na^+ concentration 0.8 mM) followed by a dietary intervention or diuretic treatment for another 5 days. All metabolic cage experiments were preceded by a 24h run-in period and meticiolous care was taken to avoid urinary losses (i.e. the collecting apparatus was siliconized to ease dripping of urine into the urinary flasks; urine was collected beneath water saturated oil to prevent evaporation and a specially designed metal grid was used to prevent fecal contamination). The mice were subjected to a low Na^+-normal K^+ diet (C1036, Na^+ content 6 µmol/g, K^+ content

182 µmol/g), a normal Na^+-high K^+ diet (C1051 modified, Na^+ content 117 µmol/g, K^+ content 1281 µmol/g) or a low Na^+-high K^+ diet (C1058, Na^+ content 6 µmol/g, K^+ content 1281 µmol/g all Altromin, Lage, Germany). A high- Na^+-high K^+ diet was achieved by giving C1051 as food with 0.45 % NaCl (77 mM) in the drinking water. Diuretics were supplied in the drinking water without any electrolyte supplementation. Furosemide (125 mg/l) was applied to block NKCC2 in the loop of Henle, hydrochlorothiazide (400 mg/l) was applied to block NCC in the distal convoluted tubule, triamterene (200 mg/l, pH 3) was applied to block the epithelial sodium channel (ENAC) in the late distal convoluted tubule and collecting duct (all diuretics Sigma, Schnelldorf, Germany) (4). Collections were performed each day at 9 am, hence all time-dependent values were acquired over 24h. Blood samples were drawn prior to and at the end of the different treatments by puncturing the right retro-orbital plexus under isoflurane anesthesia.

Blood and urine measurements

Plasma and urinary concentrations of Na^+ and K^+ were measured by flame photometry (efux 5057, Eppendorf, Hamburg, Germany). Urinary creatinine concentration was measured using the Jaffé method, plasma urea concentration was measured using an enzymatic method (Lehmann, Berlin, Germany). Plasma aldosterone concentration was measured using a RIA kit (Demeditec, Kiel, Germany).

Cell culture

Primary culture of distal tubular segments was done after collagenase II (Worthington, Lakewood, NJ, USA, 75U/ml), Pronase E (Sigma, Schnelldorf, Germany, 1.25 U/ml) and DNaseI (Applichem, Darmstadt, Germany 50U/ml)

perfusion of mice kidneys followed by a digest of renal slices and dissection of GFP positive tubuli using a Leica Fluorescent-Dissection Microscope (Leica, Wetzlar, Germany). After dissection tubular segments were either snap frozen for subsequent western blot analysis or cultured in supplemented DMEM 1:1 HAMS F12. After 9 to 10 days, once confluency was reached, cells were sorted using a BD ARIA FACS (BD Bioscience, Heidelberg, Germany). Briefly, after using TrypLE Express (Life Technologies, Karlsruhe, Germany) to dissociate cells, these were poured through a 40 nm mesh. Single cells were sorted using a 100 µm nozzle, a sheet pressure of 20 PSI and a laser excitation of 488 nm. For genomic and proteomic analysis only GFP positive cells were used. M1 cells (ATCC, Manassas, VA, USA M1 CRL-2038) were cultured using DMEM supplemented with FCS. Cells were grown to confluency before start of the respective experiment which either added aldosterone (final concentration 1µM) for 0, 6, 12 and 24h or increased K^+ in the cell culture medium from 5.3 to 7.3; 8.8 and 10.3 mM.

Genomic knock-out proof

DNA was extracted using hot-shot alkaline extraction buffer. The following primers were used: common forward TTA TTA ACT GTG TGT GGG TTG, reverse wild-type CGT CTT AGT GTT GCT GTC TAG (expected product length 197 bp), reverse knock-out CAG ATT CAA GCA TGT CCT AAG C (expected product length 280 bp).

Western Blot

Kidneys, macroscopically divided into cortex and medulla or isolated tubular segments were glass-glass-homogenized in lysis buffer for 2 min (modified RIPA Buffer containing: 50mM Tris/HCl pH 7,5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 0,1 % (w/v) SDS, 50 mM NaF, 150 mM NaCl, 0,5% (w/v) Na-Deoxycholate,

0,1 %(v/v) 2-mercaptoethanol, 1mM Na-Orthovanadate, Roche Ultra complete proteinase inhibitor cocktail and Roche Phospho-STOPP as indicated by the manufacturer (Roche, Mannheim ,Germany) and ddH_20 ad final volume; 15µl lysis buffer per 1 mg of tissue were used). After incubation on ice for 15 min the lysates were centrifuged (1000 x g, 5 min, 4°C), the supernatant was recovered and the protein concentration was determined by DC Protein-Assay (Bio-Rad, Munich, Germany). Samples were heated after addition of 2x Laemmli buffer (inlcuding 100 mM DTT) at 42°C for 30 min. Cortical lysates were used to examine RICTOR expression. Equal amounts of protein (80 µg per lane) were separated on SDS page. HRP coupled 2^{nd} antibodies and ECL in combination with a conventional x-ray system (films: Fuji, Tokio, Japan; developer: AGFA, Mortsel, Belgium) were used to detect western blot bands. Western blots were densitometrically analyzed using LabImage software (Labimage Software, Leipzig, Germany). Only bands showing no overexposure were used for further analysis. Relative intensities giving ratios of respective protein band intensity to loading control band intensity of the same blot are shown.

Immunofluorescence and PAS staining of kidney sections

Kidneys were frozen in OCT compound and sectioned at 5 µm (Leica Kryostat, Wetzlar, Germany). The sections were fixed with 4% paraformaldehyde or Methanol, blocked in PBS containing 5% BSA + 5% Normal Donkey Serum and incubated for 45 min with primary antibodies as indicated. After several PBS rinses, fluorophore-conjugated secondary antibodies (LifeTechnology, Karlsruhe, Germany) were applied for 30 minutes. Images were taken using a Zeiss fluorescence microscope equipped with a 20x and 63x water immersion objective (Zeiss, Oberkochen, Germany). For PAS staining, kidneys were fixed in 4% paraformaldehyde in PBS,

embedded in paraffin and further processed by standard procedures. Quantification of immunofluorescence was done using the Image J software package. Integrated density and corrected total cell fluorescence (CTCF) of alpha-BK in at least 15 individual intercalated cells per 20x picture were measured on eight-bits images of three animals of each genotype for both LS/HK and triamterene.

Primary and Secondary Antibodies

The following primary antibodies were used: anti RICTOR (Cell Signaling CS9476, Danvers, MA, USA); anti RICTOR (Biorbyt Limited orb 37673, Cambridge, UK), anti RICTOR (Abcam ab70374, Cambridge, UK); anti α-ENAC (StressMarq SPC-403D, Victoria, BC, USA), anti ß-ENAC (StressMarq SPC-404D), anti y-ENAC (StressMarq SPC405D) (5); anti P-Ser 422 SGK1 (Santa Cruz sc-16745, Dallas, Tx, USA), anti P-Ser657-PKCalpha (Santa Cruz sc-12356), anti BK α (Alomone Labs, APC-107, Jerusalem, Israel), anti Ki67 (Thermo Scientific, Waltham, MA, USA), anti ROMK (Atlas Antibodies HPA026962, Sigma, Schnelldorf), anti AQUAPORIN 2 (Alomone Labs, AQP-002), anti AQUAPORIN 2 (Santa Cruz sc-9882), anti CALBINDIN D28K (Swant, Bellinzona, Switzerland), anti DESMIN (Sigma D1033), anti y-TUBULIN (Sigma SAB4503045), anti NCC (StressMarq SPC402D), anti P-T58 NCC (generous gift S. Bachmann, Charité, Berlin, Germany) (6) and anti α-ACTININ 4 (Santa Cruz sc-17829). Secondary HRP coupled antibodies were bought from Cell Signaling and secondary Alexa coupled antibodies were purchased from Life Technologies.

Patch-clamp Experiments

Solutions: Na^+-rich bath solution contained (in mM) 145 Na gluconate, 5 K gluconate, 2 $CaCl_2$, 5 barium acetate, 1 $MgCl_2$ and 5 HEPES (pH adjusted to 7.4 with Tris). K^+-

rich bath solution contained (mM): 110 K gluconate, 12 Na gluconate, 2 $CaCl_2$, 1 $MgCl_2$, 45 NMDG gluconate, 5 HEPES (pH adjusted to 7.4 with gluconic acid). Ba^{2+} containing K^+-rich solution was identical to the previous one except that it was supplemented with 5 mM of Ba^{2+} acetate and contained only 40 mM instead of 45 mM NMDG gluconate.

Preparation of renal tubules: Tubular CNT/CCD fragments were prepared as previously described (7). In this nephron segment ENaC regulation is aldosterone-dependent. After anesthesia [Ketamine (100 mg/kg), CP-Pharma Burgdorf, Germany and Rompun (10 mg/kg) Bayer, Leverkusen, Germany], the circulatory system was perfused via the left ventricle with Leibovitz medium (LM), containing collagenase (1 mg/ml) and amiloride (2 µM). After perfusion, both kidneys were removed, cut into coronal slices, and incubated for 20–25 min at 37° C in the same solution as used for the perfusion. Following collagenase wash out, slices were kept in ice-cold LM containing amiloride (2 µM) throughout the microdissection procedure. Cortical tubules were separated manually using fine forceps. We identified and isolated tubular segments with characteristic branching indicative of the merging of CNT into CCD and extended the dissection towards the CCD. To gain access with the patch pipette to the apical cell membrane, tubules were cut open with a broken glass pipette attached to a micromanipulator. Cells, which were affected by the mutation, were identified by GFP fluorescence, only fluorescent cells were patched. Experiments were performed in tubules obtained from *Rictor fl/fl*Ksp*Cre mice or from *KspCre* control animals.

Electrophysiology: A computer-controlled EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) was used to perform conventional whole-cell and outside-out patch clamp recordings from principal cells in microdissected tubules essentially as described earlier (7). Pipettes were made from borosilicate glass

(Hildenberg, Masfeld, Germany) with a resistance of about 4.5-5 MΩ. Experiments were performed at 37 ºC. Seals were formed at the apical cell surface by using gentle suction. The whole-cell configuration was achieved by increasing the suction after the seal resistance had exceeded at least one GΩ. Series resistance ranged between 10 and 15 MΩ, and was not compensated. For continuous whole-cell as well as outside-out current recordings, cells were voltage clamped using a pipette holding potential (V_{hold}) of -60 mV. Data were sampled at a rate of 4 kHz and initially filtered at 1 kHz. For further analysis, they were routinely re-filtered at 200 Hz. Whole-cell current recordings were started in Na^+-rich solution containing 2 µM of amiloride. Subsequently, amiloride was washed out and reapplied. As a measure of ENaC activity the amiloride-sensitive whole-cell current (ΔI_{Amil}) was determined by subtracting the whole-cell current in the presence of amiloride from that measured in its absence. After determining ΔI_{Amil} in the whole-cell configuration, outside-out patches were excised and the Na^+-rich bath solution was replaced by Ba^{2+}-containing K^+-rich bath solution. To estimate K^+ channel activity in the apical membrane the Ba^{2+}-sensitive current component was determined in outside-out patches by washout and reapplication of Ba^{2+}. Single channel currents were estimated from binned current amplitude histograms; voltage step protocols from -100 mV to +40 mV were used to determine the single channel current-volatage relationship of barium-sensitive K^+-channels. Outward conductance (pS) was calculated as

$$g_{out} = \frac{i_{(-100)} - i_{(-60)}}{40\ mV} \cdot 10^3$$

and inward conductance as

$$g_{in} = \frac{i_{(40)}}{40\ mV} \cdot 10^3$$

Where $i_{(-100)}$, $i_{(-60)}$, $i_{(40)}$ are single channel currents (pAmp) at the corresponding potentials (mV).

Statistics

Data are expressed as mean ± SEM or as median, lower and upper quartiles, when the distribution was far from normal. Statistical comparisons were performed using the GraphPad Prism Software Package 6 (GraphPad Software Inc., LaJolla, USA) with two-tailed Student's t-test or ANOVA including respective corrections where indicated. Differences with p values greater 0.05 were considered significant. For patch-clamp experiments data are given as lower (Q25), median (Q50) and upper quartiles (Q75) because of the highly asymmetric character of the observed current distributions; the non-parametric Kolmogorov-Smirnov test was used to test differences for significance.

Study approval

All animal experiments were approved by local authorities [G-10/39 (Regierungspräsidium Freiburg), M 9/11 (Regierungspräsidium Tübingen), 621.2531.32-2/05 (Bezirksregierung Mittelfranken, Ansbach)].

Supplementary Figure Legends

Suppl. Figure 1: Confirmation of distal tubular mTORC2 deletion, RICTOR expression in the distal tubule and lack of obvious phenotype of Rictor fl/fl*KspCre under physiological conditions. (A) FACS sorted primary tubular cells of *Rictor fl/fl*KspCre* mT/mG* and *Rictor fl/fl* mT/mG* mice were used for western blotting. A second anti-RICTOR (ab70374) antibody was used to confirm the results obtained in Fig. 1 B (CS 9476). RICTOR Protein is virtually absent in isolated primary cells of *Rictor fl/fl*KspCre* mT/mG* mice. (B) Knock-out on a genomic level in primary isolated tubular cells was confirmed by RT-PCR, proving the findings obtained in Fig. 1 B and Suppl. Fig. 1 A. (C, D) Expression of *Ksp* driven *Cre* is restricted to TAL, DCT and CD, while proximal tubules and glomerula do not show any *Cre* expression (*Cre* expressing tissues - green, non-*Cre* expressing tissue - red, nuclei - blue Hoe 33342; representative images of n=3 examined animals are shown). (E) RICTOR expression after 5 days of triamterene is most prominent in DBA positive tubular segments (red – RICTOR, green – DBA, blue Hoe33342). (F) Expression in TAL as marked by THP (green) under these conditions is rather low (representative images of n=3 examined animals are shown). (G, H) At one year of age histology is normal in *control* (G) and *Rictor fl/fl*KspCre* animals (H) as was assessment of fibrosis (green - DESMIN, red – THP, blue Hoe33342) (I, J) and proliferation (green- KI67, red - THP, blue - Hoe33342; representative images of n=3 examined animals per genotype are shown) (K, L) in unstressed conditions. (M - Q) Main functional parameters were not different between *control* and knock-out mice (control = white bars, knock- outs = black bars, Student's t-test; n=5-30).

Suppl. Figure 2: K^+ dependent regulation of RICTOR in a mouse collecting duct cell line. (A – C) Interestingly, using the mouse collecting duct cell line M1 we can show that not aldosterone (A) but high extracellular K^+ itself seems to be the trigger for this regulatory response in a time (B) and concentration (C) dependent manner. (D) Quantification of RICTOR Expression over time after administration of aldosterone (1µM) to M1 Cells. Compared with ethanol treated control cells (white bars) there is no significant difference in RICTOR expression levels in aldosterone treated cells (black bars) over 24h (n=4-8 independent experiments; ANOVA). (E) Incremental increase of potassium concentration in the cell culture medium leads to a robust increase in RICTOR protein levels, similar to the *in vivo* situation after triamterene administration (n=6 independent experiments; ANOVA; # $p < 0,05$).

Suppl. Figure 3: Expression and localization of α-, β-, γ-ENaC, NCC, P-T58 NCC and α-BK in untreated control and Rictor fl/fl*Ksp Cre animals.

(A-X) Untreated animals did not exhibit any difference in localization or qualitative abundance of α-, β-, γ-ENaC (A – L), NCC (M – P), P-T58 NCC (Q – T) or α-BK (U – X) proteins between genotypes. (red – respective transporter, channel or channel subunit as indicated, [A – L, U – X] green - AQP 2, [M – T] green – CALBINDIN D28K, blue – Hoe 33342; b/w image is the signal of the given red channel only; representative images of n=3 examined animals per genotype are shown).

Suppl. Figure 4: Expression and localization of α-, β-, γ-ENaC, NCC, P-T58 NCC and α-BK in LS treated control and Rictor fl/fl*Ksp Cre animals.

(A-X) Animals given LS diet did not exhibit any discernible difference in localization or qualitative abundance of α-, β-, γ-ENaC (A – L), NCC (M – P), P-T58 NCC (Q – T) or α-BK (U – X) proteins between genotypes. (red – respective transporter, channel or

channel subunit as indicated, [A – L, U – X] green - AQP 2, [M – T] green – CALBINDIN D28K, blue – Hoe 33342; b/w image is the signal of the given red channel only; representative images of n=3 examined animals per genotype are shown).

Suppl. Figure 5: Expression and localization of α-, β-, γ-ENaC, NCC, P-T58 NCC and α-BK in LS/HK treated control and Rictor fl/fl*Ksp Cre animals.

(A-L) While localization and qualitative abundance of α-, β-, γ-ENaC and NCC (M – P) did not appear to be decisively changed, the apical membrane localization and abundance of P-T58 NCC (red arrowheads; Q – T; asterisk indicates vessel) was greatly reduced as was the number and staining intensity of α-BK positive intercalated cells (red arrowheads, U – X) (red – respective transporter, channel or channel subunit as indicated, [A – L, U – X] green - AQP 2, [M – T] green – CALBINDIN D28K, blue – Hoe 33342; b/w image is the signal of the given red channel only; representative images of n=3 examined animals per genotype are shown).

Suppl. Figure 6: Expression and localization of α-, β-, γ-ENaC, NCC, P-T58 NCC and α-BK in triamterene treated control and Rictor fl/fl*Ksp Cre animals.

Animals treated with triamterene did exhibit a difference in localization and qualitative abundance of β- and γ-ENaC (E – L), P-T58 NCC (Q – T) and α-BK (U – X). α-ENaC (A – D) and NCC (M – P) were not different between genotypes. Interestingly β- and γ-ENaC also exhibited a strong signal in non-AQP 2 positive segments of the distal tubule (red arrowheads in H, L, T, X; red – respective transporter, channel or channel subunit as indicated, [A – L, U – X] green - AQP 2, [M – T] green – CALBINDIN

D28K, blue – Hoe 33342; b/w image is the signal of the given red channel only; representative images of n=3 examined animals per genotype are shown).

Suppl. Figure 7: Single channel analysis of obtained patches implies ROMK as the detected channel. (A) Representative traces of Ba^{2+}-sensitive channels recorded in K^+-rich bath solution (V_{hold}=-60 mV) from an outside-out patch obtained from a split open tubular segment from the CNT/CCD transition zone of a control animal. The recording was obtained about three minutes after patch excision when spontaneous channel run down permitted the resolution of single channel events. (B). Representative current recordings of Ba^{2+}-sensitive channels at different holding potentials from the same experiment as shown in E. (C) Current-voltage relationship of single-channel currents obtained from the recordings shown in B. Inward and outward single channel conductance was calculated to be 41 pS and 17 pS respectively (n= 10-24).

Suppl. Figure 8: α-BK is reduced in intercalated cells of Rictor fl/fl*KspCre mice under LS/HK and triamterene. Corrected Total Cellular Fluorescence (CTCF) was used to determine the protein abundance of α-BK in intercalated cells on the collecting duct of LS/HK and triamterene treated control and *Rictor fl/fl*KspCre* animals. Under LS/HK CTCF was reduced by approximately 50% in *Rictor fl/fl*KspCre* compared with control animals while under triamterene CTCF was reduced even more in *Rictor fl/fl*KspCre* mice (control – white bars, *Rictor fl/fl*KspCre* – black bars; Student's t-test; ### p< 0.001, three animals per condition, at least 15 individual intercalated cells analyzed per animal)

Supplementary References

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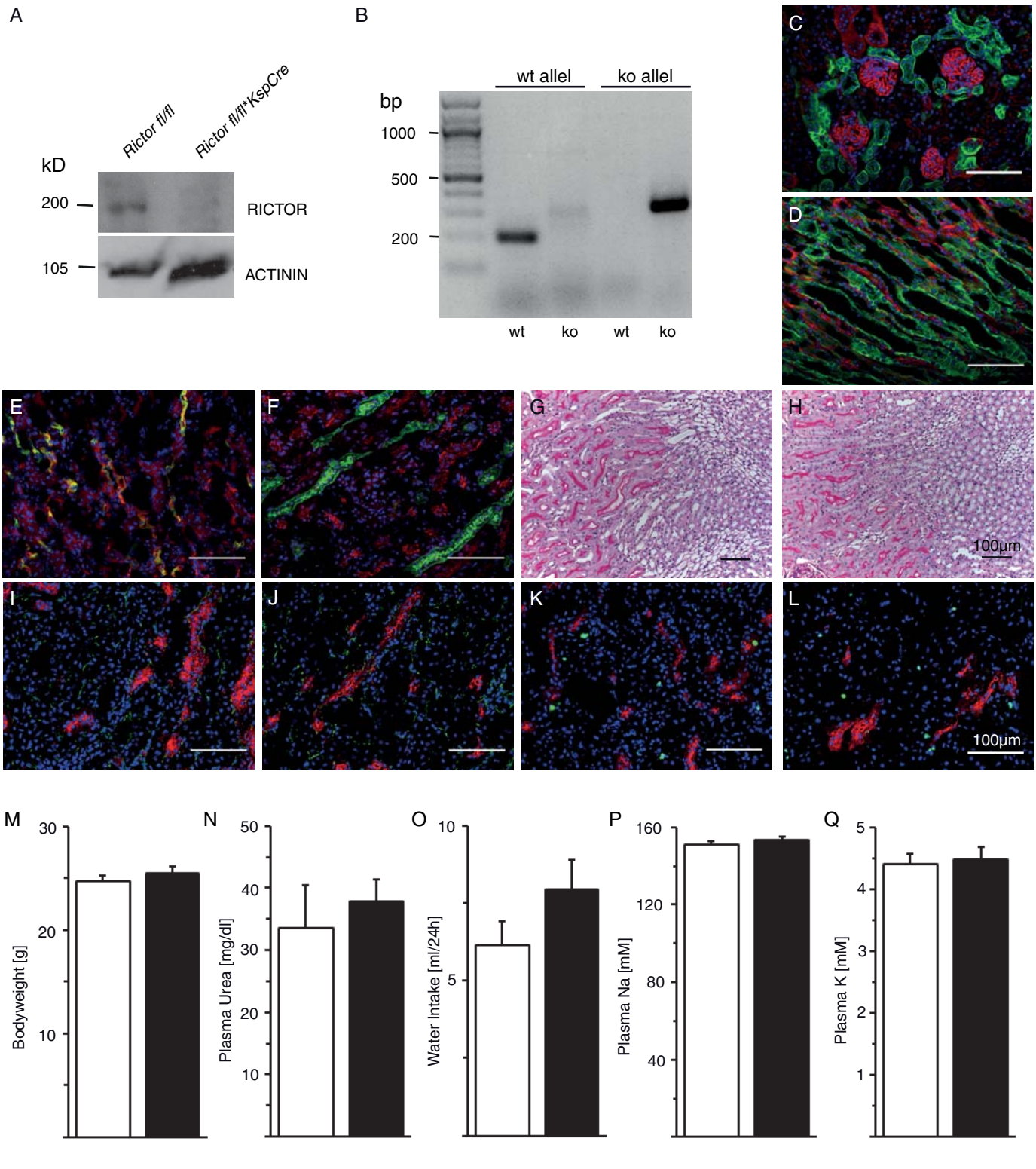
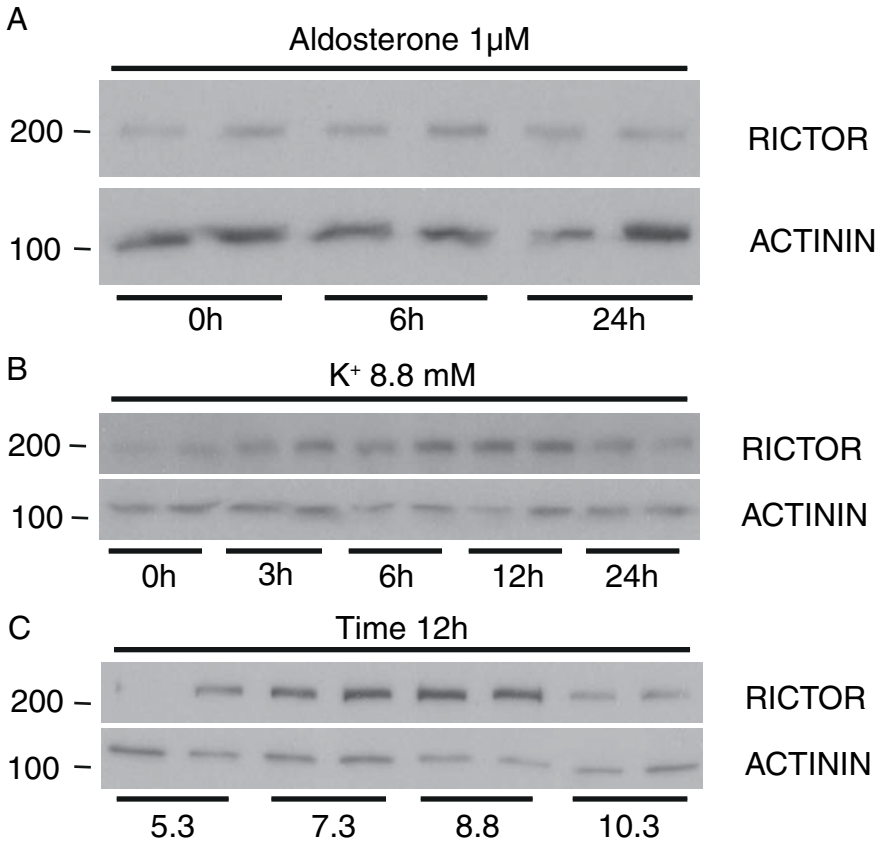
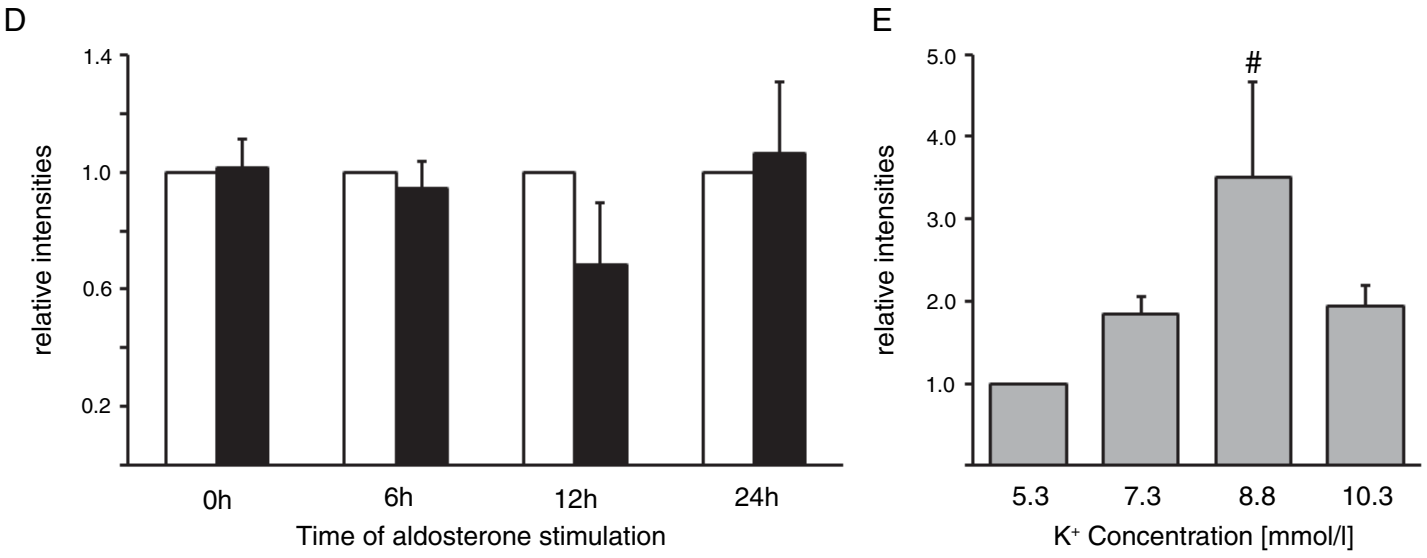
Suppl. Figure 1

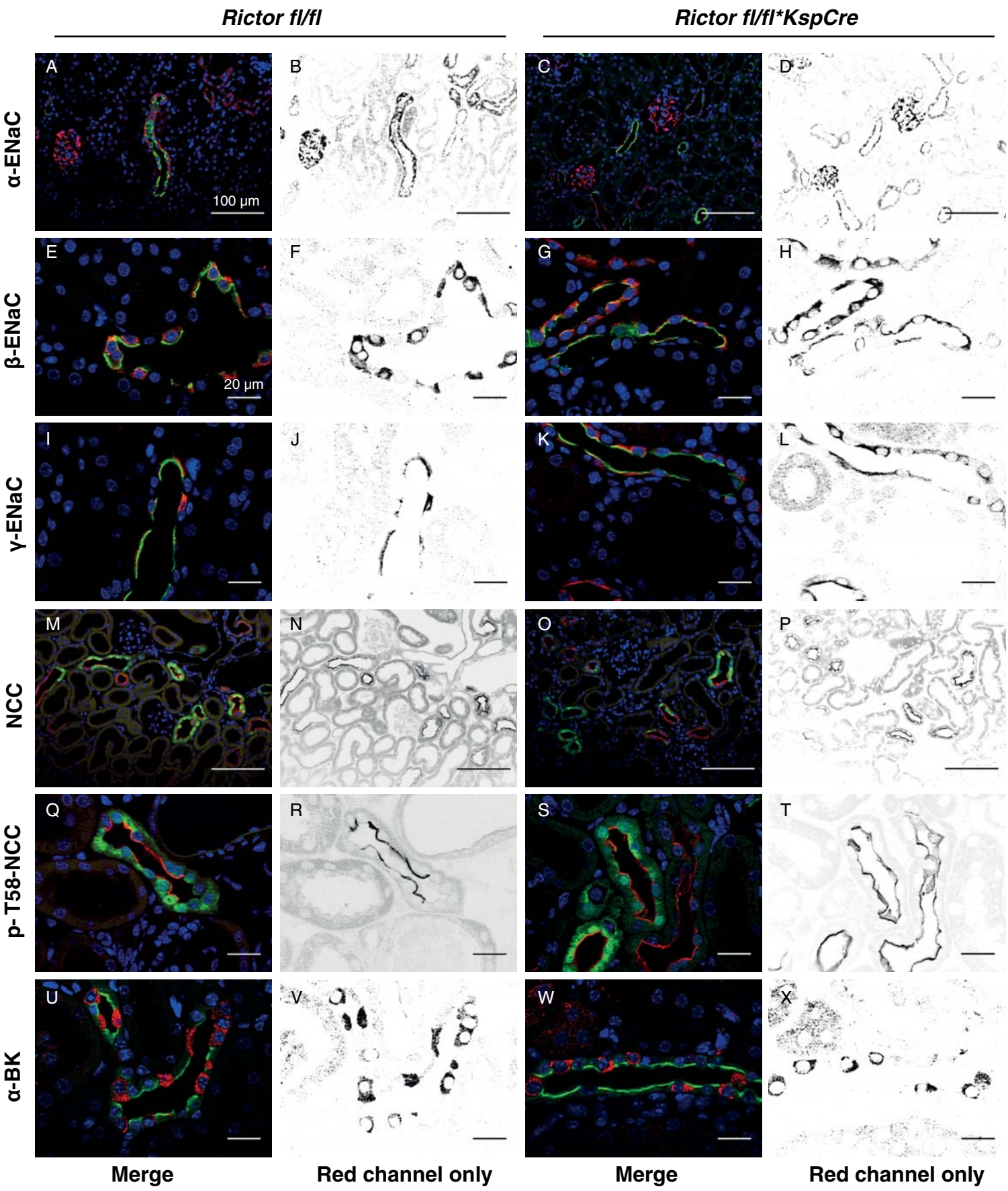


Suppl. Figure 2





Suppl. Figure 3: Untreated



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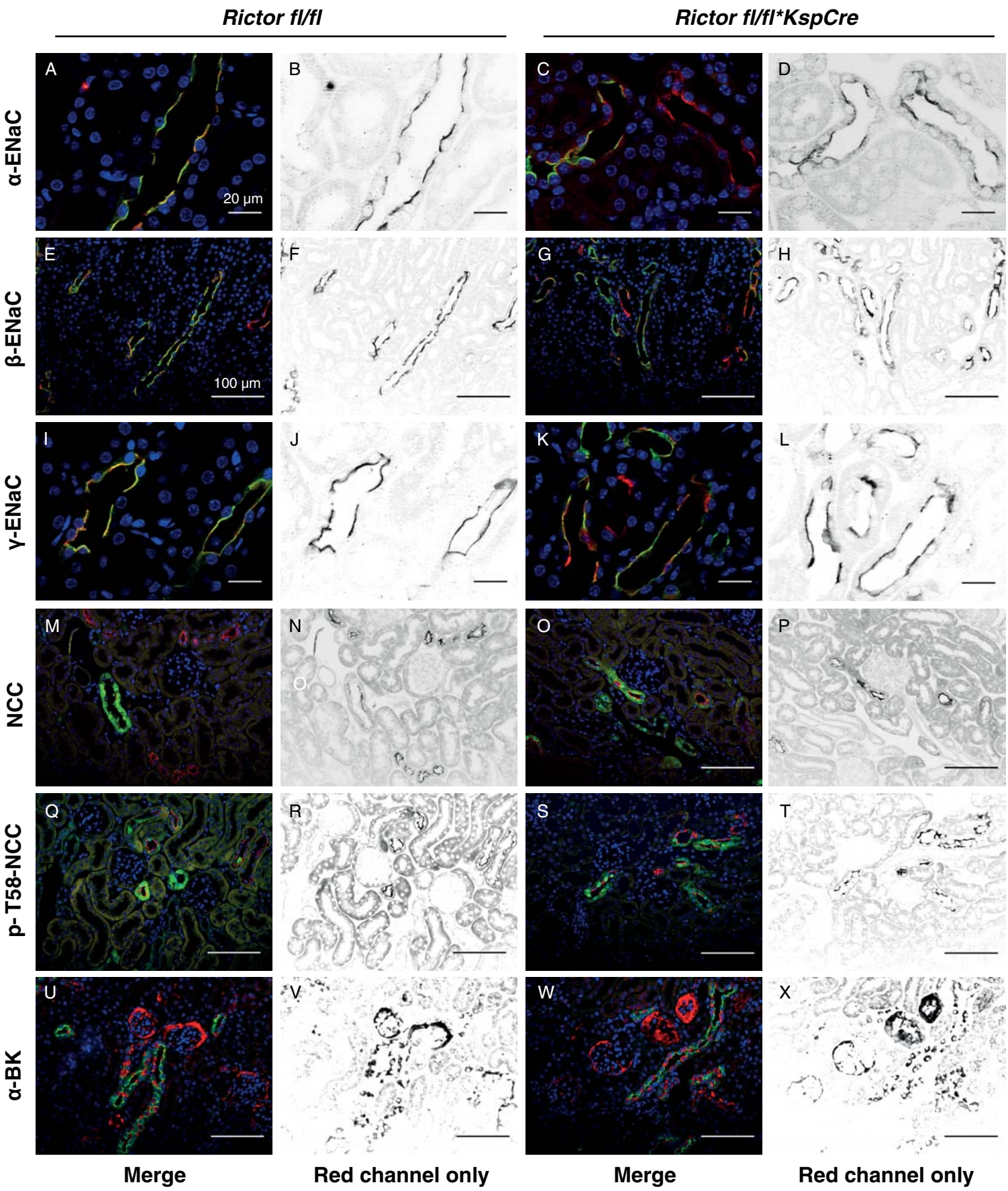

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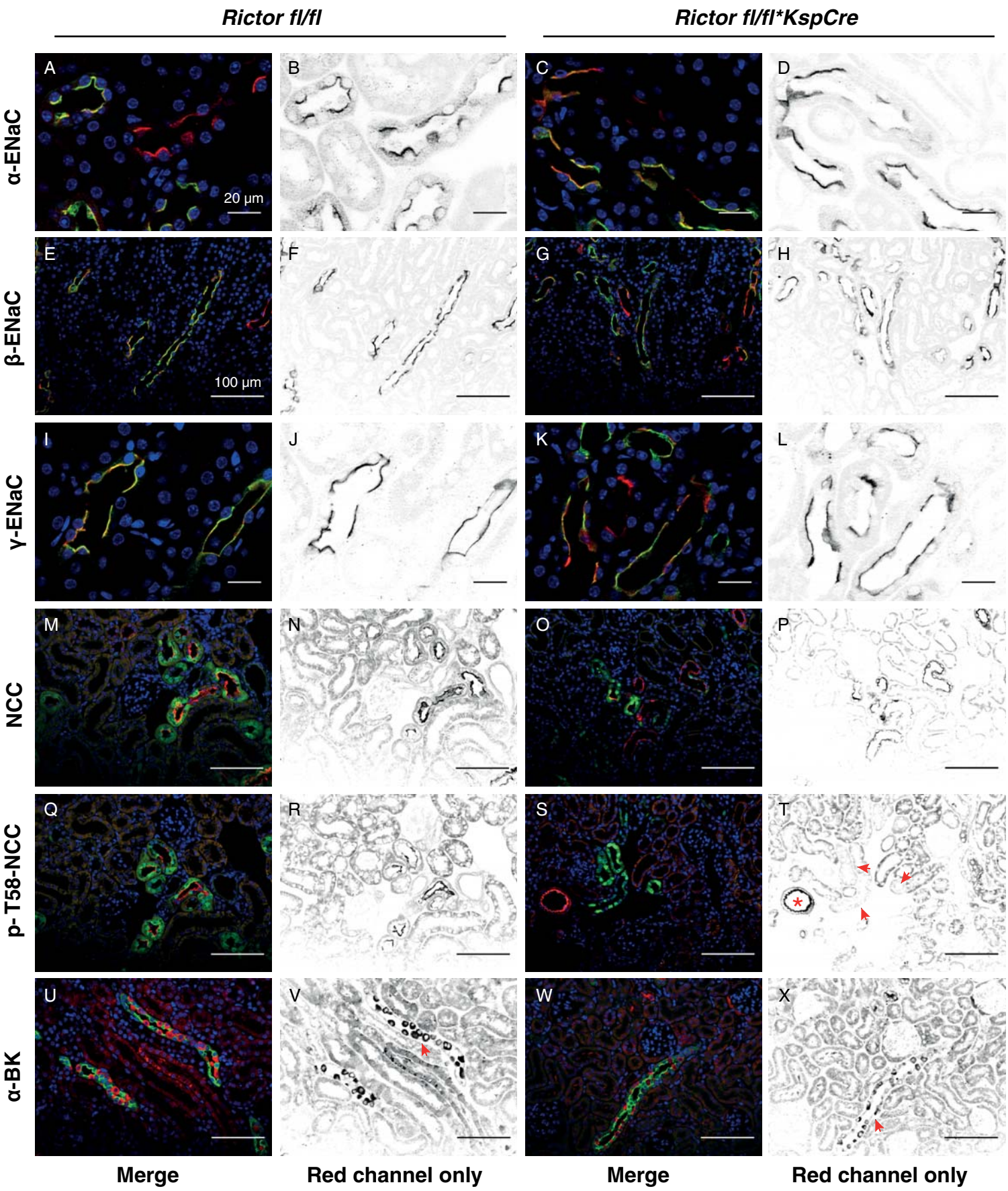
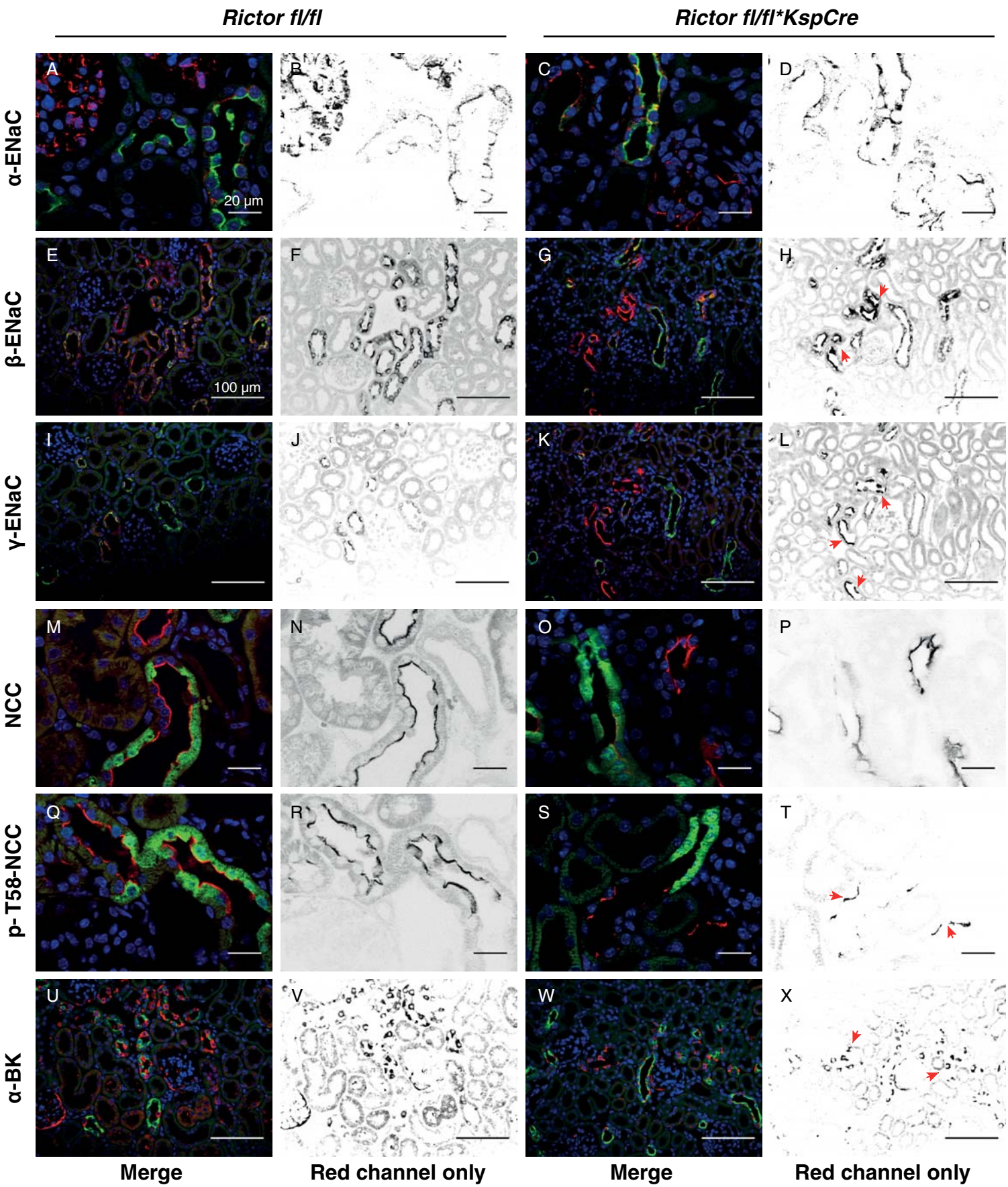
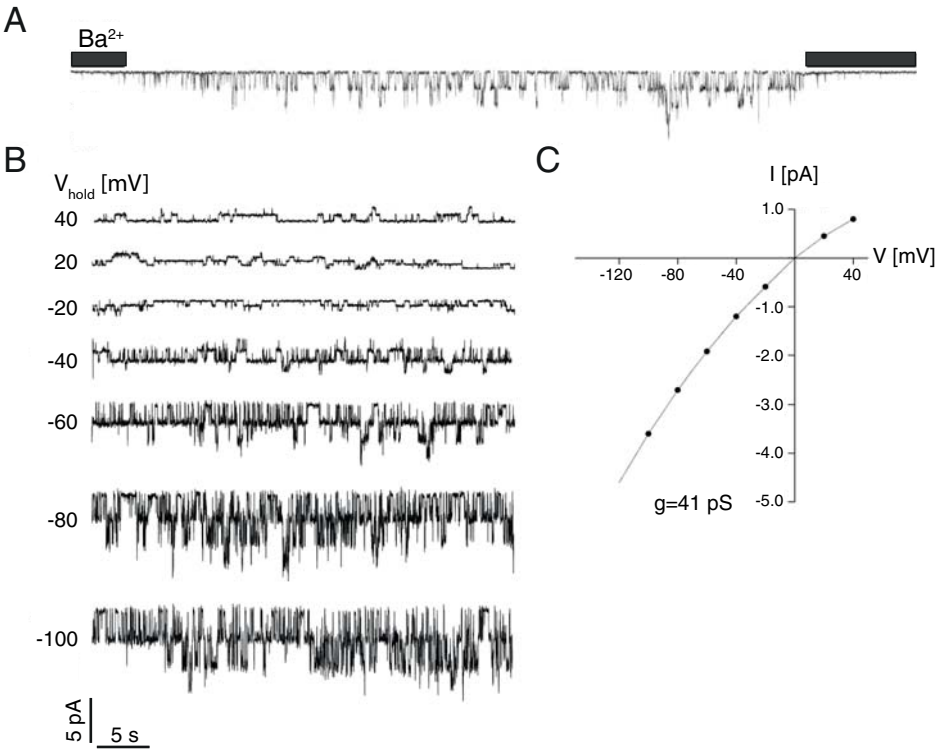


Suppl. Figure 6: Triamterene



Suppl. Figure 7



Suppl. Figure 8